Cancer Immunol Immunother (1998) 46: 253–260

ORIGINAL ARTICLE

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Stimulation of immune suppressive CD34+ cells from normal bone marrow by Lewis lung carcinoma tumors

Received: 8 December 1997 / Accepted: 27 February 1998

Abstract Progressive growth of metastatic Lewis lung carcinoma (LLC-LN7) tumors is associated with increased levels of bone-marrow-derived CD34+ cells having natural suppressor (NS) activity toward T cells. The present studies determined whether tumor-derived products are responsible for this induction of NS activity. Culturing normal bone marrow cells with LLC-LN7-conditioned medium (LLC-CM) or with recombinant granulocyte/macrophage-colony-stimulating factor (GM-CSF) resulted in the appearance of NS activity. The development of NS activity coincided with a prominent increase in the levels of CD34+ cells. That the CD34+ cells were responsible for the NS activity of the bone marrow cultures containing LLC-CM was shown by the loss of NS activity when CD34+ cells were depleted. The stimulation of CD34+ NS cells by LLC-CM was attributed to tumor production of GM-CSF, since neutralization of GM-CSF within the LLC-CM reduced its capacity to increase CD34+ cell levels. Studies also showed that the induction of CD34+ NS cells by LLC-CM and GM-CSF could be overcome by including in the cultures an inducer of myeloid differentiation, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. These results demonstrate that the mechanism by which the LLC-LN7 tumors stimulate increased levels of CD34+ NS cells from normal bone marrow is by their production of GM-CSF and that this can be blocked with the myeloid differentiation inducer 1,25$(OH)_2D_3$.

Key words Natural suppressor cells · Immune suppressor cells · CD34+ cells · Tumor · Cancer

This work was supported in part by the Medical Research Service of the Department of Veterans Affairs, and by grants CA-45080 and CA-48080 from the National Institutes of Health

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Introduction

Cancers are vulnerable to immune effector cells such as macrophages, lymphokine-activated natural killer cells and tumor-specific cytotoxic T lymphocytes [8, 18, 34]. However, the functions of lymphocytes, including those of tumor-infiltrating lymphocytes, are suppressed in cancer patients and in animal tumor models [1, 2, 25, 28, 45]. While tumors can directly inhibit immune function by producing soluble immune inhibitory factors, they can also stimulate the increased appearance of immune suppressor cells. Such suppressor cells include suppressor T cells or macrophages [3, 4, 14, 22, 29]. In addition, tumors can induce suppressor cells that are not typical T cells or macrophages. Instead, these suppressor cells are a less mature population that exhibits natural suppressor (NS) activity, which is described as non-specific suppression of immune function [17, 26, 32, 42, 43].

NS cells have been described in human cancers, animal tumor models, in non-tumor models where myelopoiesis is stimulated, and in normal human bone marrow [5, 7, 11, 13, 23, 24, 32]. Our studies and those of others have shown that tumor production of granulocyte/macrophage-colony-stimulating factor (GM-CSF) correlates with an increased presence of NS cells [26, 36, 42]. Many tumor types, such as human prostatic, ovarian, lung, and head and neck cancers, and an equally broad spectrum of murine tumors, express GM-CSF mRNA and protein [6, 9, 16, 27, 30, 39, 42]. Using the murine metastatic Lewis lung carcinoma (LLC-LN7) tumor model, we previously showed that NS cell levels increase during progressive tumor growth, appearing first in the bone marrow, and then also in the spleen and within the tumors [31, 36, 43]. Similar NS cells were shown to be within human head and neck squamous cell carcinomas (HNSCC) cancers and in the peripheral blood of patients with these cancers [12, 42]. The NS cells in HNSCC patients as well as those in the LLC-LN7 tumor model have been identified as CD34+ granulocyte/macrophage progenitor cells having the capacity to

develop into mature progeny such as granulocytes, monocytes or dendritic cells [12, 36, 43].

While our studies with murine and human tumors had shown that tumor production of GM-CSF correlates with the appearance of CD34+ NS cells, it has been assumed that these CD34+ NS cells develop as a result of the tumor-derived GM-CSF. The present studies aimed to determine if tumor-derived products are sufficient to induce the expansion of bone marrow-derived CD34+ NS cells and whether the proliferative signals of the tumor-derived products can be overcome so as to stimulate differentiation of the CD34+ NS cells into nonsuppressive progeny.

Materials and methods

LLC-LN7 tumor cells

Studies used a cloned LLC-LN7 tumor line that was originally derived from a metastatic lung nodule of a mouse bearing a s.c. LLC tumor. These cells were cultured in RPMI-1640 medium containing 10% endotoxin-free defined fetal bovine serum as previously described [43]. Conditioned medium from the LLC-LN7 cells (LLC-CM) was obtained from 24-h cultures of approximately 5×10^5 LLC-LN7 cells/ml. For studies requiring tumor-bearing mice, 10^6 LLC-LN7 cells were implanted by s.c. injection into 8-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Me.). After 3 weeks of tumor growth, mice were sacrificed, and bone marrow cells were collected and used as described below.

Bone marrow cultures

Normal bone marrow cells were flushed from the femurs of 8 to 10-week-old C57BL/6 mice. After they had been distributed into a single-cell suspension, cells were counted and seeded at a density of 2×10^6/ml in either medium only, 40% LLC-CM, or 100 U/ml mouse recombinant GM-CSF. Some cultures also contained 10 nM 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$,], a concentration that we had previously found to be most effective for immune enhancement [12, 35]. In other studies, the LLC-CM were first preincubated with 4 μg/ml neutralizing anti-GM-CSF antibody or isotype control irrelevant antibody (Pharmingen, San Diego, Calif.). After 3, 8 or 12 days of culture, both adherent and nonadherent cells were collected by removing the nonadherent cells, detaching the adherent cells with a rubber scraper and pooling these adherent cells together with the nonadherent cells. Cells were counted and used for either immunostaining or for assessment of NS activity. Control cell populations included freshly collected bone marrow cells either from normal mice or from mice bearing LLC-LN7 tumors that were approximately 15 mm in diameter.

Levels of CD34+ cells

Levels of CD34+ cells within freshly isolated or cultured bone marrow cells were measured after staining by indirect immunofluorescence using ER-MP12 antibody (BMA, distributed by Accurate Chemical, Westbury, N.Y.). Bone marrow cells were incubated on ice with either the ER-MP12 antibody or isotype control for 45 min, washed, and then additionally incubated for 45 min with fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-(rat Ig) antibody. The cells were then washed and the percentage of positive-staining cells was measured in a FACS 420 flow cytometer (Becton Dickinson, Sunnyvale, Calif.). Data are shown as either histograms or as the percentage positive-staining cells ± SD of at least five determinations.

Assay to measure the NS activity of bone marrow cells

The NS activity of bone marrow cells was measured by the capacity to inhibit the activation of normal spleen cells in response to stimulation through the TCR/CD3 complex. The bone marrow cells used for measurement of NS activity were first irradiated (25 Gy), adjusted to an equal number of viable cells, and then added at various ratios to 10^5 normal spleen cells in microtiter wells that had been coated with anti-CD3 antibody (PharMingen). Controls included either the spleen cells or bone marrow cells alone. After 5 days of incubation at 37 °C, supernatants were collected from these cultures for assessment of whether the bone marrow cells inhibited T-cell stimulation to release interferon γ (IFNγ). IFNγ in the supernatants was measured by enzyme-linked immunosorbent assay (Endogen, Boston, Mass.). The cells that remained in the microtiter wells were used to measure whether the bone marrow cells inhibited T cell proliferation in response to TCR/CD3 stimulation. Proliferation was measured with a tetrazolium-based colorimetric assay in which the colored formazan product is directly proportional to the number of cells (CellTiter96 AQ assay; Promega Corp., Madison, Wis.). T cell blastogenesis was calculated as the absorbance of the experimental cultures minus the absorbance of either the background medium or the irradiated suppressor cells only. Data are shown as means ± SEM from at least four separate experiments, each having triplicate determinations.

In some studies, the bone marrow cultures of CD34+ cells were first depleted before the NS activity of the culture was assessed. This was accomplished immunomagnetically by first mixing bone marrow cells with ER-MP12 antibody for 30 min on ice. After washing, the CD34+ cells were isolated with anti-immunoglobulin-coated magnetic beads (Miltenyi Biotec, Auburn, Calif.). The remaining cells were used as the CD34- cell fraction. The capacity of these cell fractions to inhibit T cell proliferation was then measured as described above. The efficiency of CD34+ cell depletion of the bone marrow cells was verified by incubating the cells overnight, washing and staining these cells by indirect immunofluorescence with the ER-MP12 antibody and FITC-conjugated anti-(rat Ig) antibody. The cells were then flow-cytometrically analyzed for positive staining. The results of six separate verifications showed that, after depletion, the proportion of contaminating CD34+ cells ranged between 0 and 4%.

Analysis of data

Student's *t*-test was used to determine the significance of differences between control and experimental values in analyses for T-cell proliferation, IFNγ production, and CD34+ cell frequencies.

Results

Increased NS activity in normal bone marrow cells after culture with LLC-CM

Whether or not soluble products produced by metastatic LLC-LN7 tumor cells could be responsible for the induction of NS activity that is typical of tumor bearers was determined. This was accomplished by culturing bone marrow cells from normal mice for various lengths of time in the presence of supernatants from LLC-LN7 cells or with control medium. Since the LLC-LN7 cells had previously been shown to produce GM-CSF (approximately 200 U/10^6 cells/24 h), bone marrow cells were also cultured with recombinant GM-CSF. The NS activity of these bone marrow cultures was then determined by irradiating the bone marrow cells, adding them at a 0.5:1 ratio to normal spleen cells, and measuring their capacity to inhibit the splenic T cell proliferative response to stimulation through

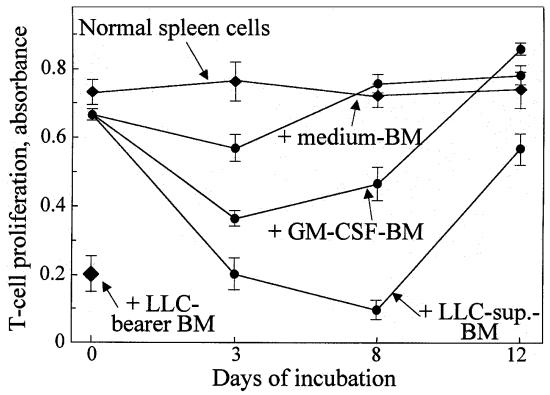

Fig. 1 Metastatic Lewis lung carcinoma conditioned medium (*LLC-CM*) stimulates the appearance of natural suppressor activity in normal bone marrow cells. Normal bone marrow cells (*BM*) were cultured for various times with LLC-CM, granulocyte/macrophage-colony-stimulating factor (*GM-CSF*) or only medium. After various times in culture, the bone marrow cells were irradiated and added at a 0.5:1 ratio to normal spleen cells. The capacity of the bone marrow cells to inhibit splenic T cell proliferation in response to immobilized anti-CD3 was measured by a tetrazolium-based colorimetric assay. Also shown are the effects of freshly isolated normal bone marrow cells and bone marrow cells from LLC-LN7-bearing mice on T cell activation. Data shown are the mean absorbances ±SEM of four separate experiments

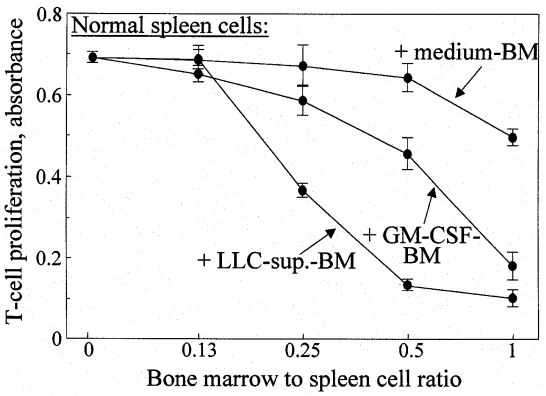

Fig. 2 Bone marrow cells that have been incubated with LLC-CM have a more potent inhibitory activity than do cells that have been incubated with either GM-CSF or medium. After 8 days of incubation of normal bone marrow cells with either LLC-CM, GM-CSF or medium, cells were irradiated, adjusted to an equal number of viable cells, and added at various ratios to a constant number of normal spleen cells. The effect of these bone marrow cells on the proliferative response of the spleen cells to stimulation through TCR/CD3 was measured colorimetrically. Data shown are the mean absorbances ±SEM of four separate experiments

the TCR/CD3 complex. In the absence of added bone marrow cells, splenic T cells proliferated in response immobilized CD3 antibody (Fig. 1). Freshly isolated normal bone marrow cells had only a minimal inhibitory effect on T cell activation. For comparison, the marked inhibitory effect ($P < 0.001$) of bone marrow cells that had been isolated from tumor-bearing mice is also shown. Culturing normal bone marrow cells for 3 days with medium only resulted in a slight appearance of suppressive activity, but this disappeared with further culture. In contrast, normal bone marrow cells became suppressive to T cell activation after 3 ($P < 0.001$) or 8 ($P < 0.001$) days of incubation with LLC-CM. By day 12 of culture, the suppressive activity had significantly subsided ($P < 0.002$ compared to day 8) to result in approximately 13% inhibition. Normal bone marrow cells also acquired NS activity after culture for 3 ($P < 0.001$) or 8 ($P < 0.01$) days with GM-CSF, although the magnitude of suppression was not as extensive as that which was induced by LLC-CM.

Shown in Fig. 2 are results of dose/response analyses in which normal bone marrow cells that had been incubated for 8 days with either medium, GM-CSF or LLC-CM were added in varying proportions to a constant number of normal spleen cells. At the highest ratio tested (1:1), the medium-cultured bone marrow cells showed inhibitory activity toward T cell proliferation in response to TCR/CD3 stimulation ($P < 0.05$). However, this NS activity was no longer evident when the medium-cultured bone marrow cells were added in lower proportions to the spleen cells. In contrast, the NS activity of bone marrow cells that were precultured with either LLC-CM or GM-CSF remained evident when lower proportions of the bone marrow cells were added to the spleen cells. The LLC-CM pre-cultured bone marrow cells showed the most potent inhibitory activity, which was detectable even at a bone marrow to spleen cell ratio of 0.25:1 ($P < 0.002$).

Whether or not LLC-CM could also induce normal bone marrow cells to inhibit T cell cytokine production was measured by testing the effect of bone marrow cells that had been incubated for 8 days with LLC-CM on T cell stimulation to produce IFNγ (Fig. 3). Splenic T cells that had been incubated on immobilized CD3 antibody produced readily detectable levels of IFNγ. This was not significantly affected by the presence of normal bone marrow cells that had been incubated with medium only. In contrast, bone marrow cells became inhibitory to T cell activation to produce IFNγ after incubation with either LLC-CM ($P < 0.01$) or with GM-CSF ($P < 0.05$). The bone marrow cells that had been preincubated with LLC-CM were slightly more inhibitory toward T cell production of IFNγ than were the GM-CSF-pretreated bone marrow cells. Shown for comparison is the minimal inhibitory effect of freshly isolated bone marrow cells and the prominent inhibition of T cell production of IFNγ by freshly isolated bone marrow cells of LLC-LN7-bearing mice ($P < 0.002$).

The sum of the results shown in Figs. 1–3 demonstrate that normal bone marrow cells are induced by LLC-LN7-derived products to become inhibitory to T cell activation. Furthermore, these results show that the suppressor-cell-stimulatory effect of LLC-CM can be mimicked in part by recombinant GM-CSF.

Increased CD34+ cell expansion from normal bone marrow cells during culture with LLC-CM

Our prior studies had shown that progressive growth of LLC-LN7 tumors results in an increased appearance of

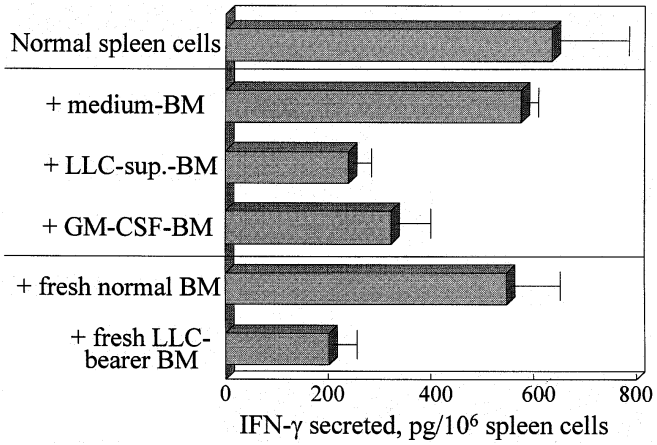

Fig. 3 Bone marrow cells that had been incubated with LLC-CM inhibit splenic T-cell production of interferon γ (IFNγ). After 8 days of incubation of normal bone marrow cells with either LLC-CM, GM-CSF or medium, cells were irradiated and added at a 0.5:1 ratio to normal spleen cells. The effect of these bone marrow cells on the stimulation of the spleen cells by immobilized anti-CD3 antibody to release IFNγ was measured. Also shown are the effects of freshly isolated bone marrow cells from normal mice and from LLC-LN7-bearing mice on normal spleen cell stimulation to produce IFNγ. Data shown are the mean absorbances ±SEM of four separate experiments

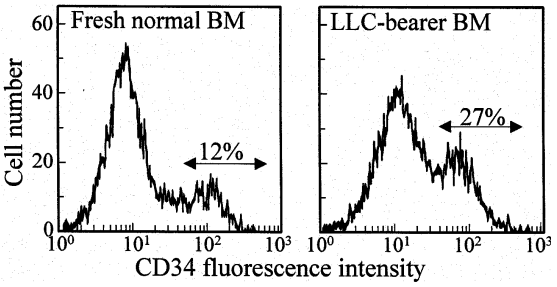

Fig. 4 Increased levels of CD34+ cells in the bone marrow cells of LLC-LN7-bearing mice. The frequency of CD34+ cells in the bone marrow from normal or LLC-LN7-bearing mice was determined by indirect immunostaining with the ER-MP12 antibody, and then analyzing the frequency of positive-staining cells by flow cytometry. Shown are histograms of CD34+ cells in bone marrow cells from normal mice (*left panel*) and from LLC-LN7-bearing mice (*right panel*). The frequency of CD34+ cells is indicated in each panel

immunosuppressive CD34+ cells first in the bone marrow and then also in the periphery [36, 40, 41]. Shown in Fig. 4 is an example of this increase in the proportion of CD34+ cells in the bone marrow of tumor-bearing mice as compared to that found in normal mice. Since the intensity of staining of bone marrow cells with the ER-MP12 antibody varies with the level of maturity of the progenitor cells [9, 20, 21], we used histograms such as those shown in Fig. 4 to establish our definition of positive-staining cells. This included cells that clearly stained positive as compared to isotype controls, without necessarily limiting these cells to being only the most brightly staining subpopulations.

On the basis of the increased levels of CD34+ cells in bone marrow of tumor-bearing mice, studies next assessed the possibility that the NS activity that was induced by LLC-CM from normal bone marrow could likewise be associated with an increase in the levels of CD34+ cells. Flow-cytometric analysis of immunostained cells showed that there was a relatively low proportion of CD34+ cells among the bone marrow cells that had been cultured for 8 days in medium only (examples from a single culture in Fig. 5, left panel; summary of six cultures in Table 1). The bone marrow cultures containing recombinant GM-CSF had an increase in the proportion of CD34+ cells (Fig. 5, right panel). The proportion of CD34+ cells was further increased in bone marrow cultures containing LLC-CM (Fig. 5, middle panel). This increase in the frequency of CD34+ cells within the LLC-CM-containing bone marrow cultures was not due to the selective decline of other cell populations since the total number of viable cells within the LLC-CM bone marrow cultures was greater than in the cultures containing GM-CSF or medium only (Table 1). By day 12 of culture, the proportion of CD34+ cells in each of the cultures had declined to where they were no longer readily detectable (not shown).

Induction of CD34+ NS cells requires tumor-derived GM-CSF

The LLC-LN7 cell tumors have previously been shown to express GM-CSF [38]. Whether or not GM-CSF is the component of LLC-CM that stimulates the increased appearance of CD34+ cells from normal bone marrow was therefore determined. Shown in Fig. 6 (left panel) is an example of the increased development of CD34+ cells from normal bone marrow cells after 8 days of culture with LLC-CM. However, when the LLC-CM was first preincubated with neutralizing antibodies against GM-CSF, its capacity to stimulate the development of CD34+ cells was markedly diminished (Fig. 6, right panel). The study shown in Fig. 6 is one of six such studies that were performed, each of which showing that GM-CSF is a required component for the LLC-CM to be able to stimulate increased levels of CD34+ cells from normal bone marrow.

CD34+ cells mediate the immune suppression in bone marrow cells that had been cultured with LLC-CM

Next it was determined whether the NS activity that was induced from normal bone marrow by the LLC-CM could be attributed to the increased numbers of CD34+ cells in these cultures. Normal bone marrow cells that had been preincubated for 3 days with LLC-CM were either unfractionated, or divided into CD34+ and CD34− cell fractions. The numbers of cells in the CD34+ and CD34− fractions were not readjusted, to test how they contributed to the NS activity of the unfractionated bone marrow cells. The capacity of these fractions to inhibit T cell proliferation in response to immobilized CD3 antibody was then measured. While the unfractionated LLC-CM-cultured bone marrow cells inhibited T cell activity, depletion of the CD34+ cells

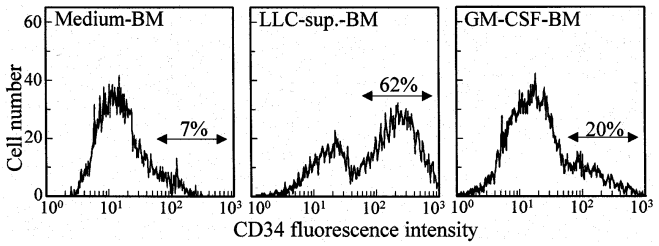

Fig. 5 Culturing normal bone marrow cells with LLC-CM increases the proportion of CD34+ cells. Normal bone marrow cells were incubated for 8 days with either medium, LLC-CM or GM-CSF. The frequency of CD34+ cells in these bone marrow cultures was determined by immunostaining with the ER-MP12 antibody, and then analyzing the frequency of positive-staining cells by flow cytometry. Shown are histograms of CD34+ cells in bone marrow cells that had been cultured with medium (*left panel*), LLC-CM (*middle panel*), or GM-CSF (*right panel*)

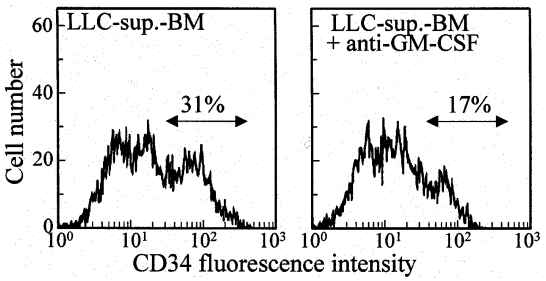

Fig. 6 Neutralization of GM-CSF diminished the capacity of LLC-CM to stimulate the increased appearance of CD34+ cells. Normal bone marrow cells were incubated for 8 days with either control LLC-CM, or with LLC-CM that had been first preincubated for 3 h with neutralizing antibodies to GM-CSF. After 8 days, the frequency of CD34+ cells in these bone marrow cultures was determined by immunostaining with the ER-MP12 antibody, and then analyzing the frequency of positive-staining cells by flow cytometry. Shown are histograms of CD34+ cells in bone marrow cells that had been cultured with LLC-CM (*left panel*), or with LLC-CM that had been incubated with anti-GM-CSF antibodies (*right panel*)

reduced the levels of NS activity from these cultures ($P < 0.02$, Fig. 7). However, the NS activity remained within the CD34+ cell fraction. These results show that a significant level of the NS activity that is induced in normal bone marrow cultures by LLC-CM is attributable to CD34+ cells. While the studies of Fig. 7 used bone marrow cells that were cultures for 3 days with LLC-CM, similar results were obtained with cells that were cultured with LLC-CM for 8 days (data not shown).

Blocking the LLC-CM-induced development of NS activity from normal bone marrow with $1,25(OH)_2D_3$

To determine if an inducer of myeloid cell differentiation can overcome the LLC-CM-mediated expansion of CD34+ NS cells, the metabolically active vitamin D_3 analog $1,25(OH)_2D_3$ was included with the LLC-CM-containing bone marrow cultures. After 8 days of incubation, the bone marrow cells containing only LLC-CM had a prominent level of NS activity (Fig. 8; $P < 0.001$ at ratios of 0.5 and 0.25). In contrast, the NS activity in cultures that also had $1,25(OH)_2D_3$ declined to essentially undetectable levels, even when the bone marrow cells were added to the spleen cells at a 1:1 ratio. Inclusion of $1,25(OH)_2D_3$ with the cultures containing normal bone marrow cells and recombinant GM-CSF similarly caused a decline in NS activity ($P < 0.01$ and 0.05 at ratios of 0.5 and 0.25 respectively). The decline in NS activity in cultures containing $1,25(OH)_2D_3$ was accompanied by a decline in the proportion of CD34+ cells to below levels of detectability (Table 1).

Discussion

Progressive tumor growth has been shown to lead to an increased appearance of bone-marrow-derived cells that are suppressive to T cell function [17, 26, 42, 43]. Among these cells having NS activity are CD34+ progenitor cells. In prior studies, it has been assumed that the increased appearance of these CD34+ NS cells occurs as a result of soluble products that are derived from the tumor cells. The present study tested this possibility by determining whether soluble products from one such tumor that induces CD34+ NS cells, the LLC-LN7 tumor, could stimulate increased levels of NS activity from bone marrow cells of normal mice.

Table 1 Metastatic Lewis lung carcinoma conditional medium (*LLC-CM*) increases the levels of CD34+ cells in normal bone marrow cell cultures; inhibition of this CD34+ cell development with $1,25(OH)_2D_3$

	Bone marrow cells incubated for 8 days with:			
Measurement	$1,25(OH)_2D_3$	Medium	LLC-CM	GM-CSF
CD34+ cells (%)	–	12 ± 2	49 ± 8	26 ± 9
	10^{-8}M	$<1\%$	2 ± 2	$<1\%$
Cell number (MTT)	–	0.38 ± 0.05	0.74 ± 0.09	0.46 ± 0.06

After 8 days of incubating normal bone marrow cells with medium, LLC-CM, or granulocyte/-macrophage-colony-stimulating factor (*GM-CSF*), in the presence or absence of 1α, 25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), CD34+ cells were immunostained with ER-MP12 antibody and the proportion of positive-staining cells determined by flow-cytometric analysis. Shown are mean absorbance values of triplicate cultures from six different experiments ±SD The number of live cells in microtiter wells was colorimetrically estimated with a tetrazolium (*MTT*) assay in which the intensity of color development is proportional to the number of live cells. Shown are mean absorbance values from six different experiments + SEM

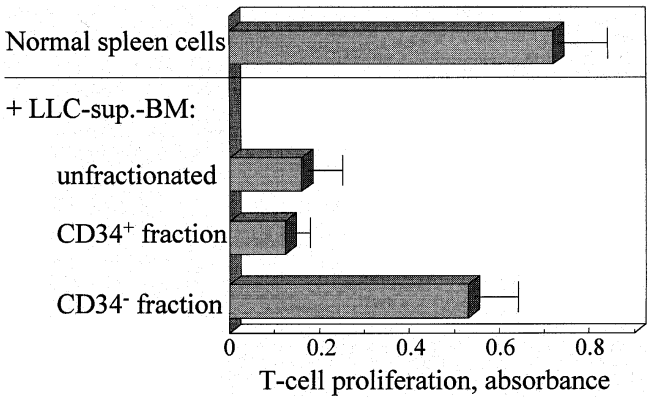

Fig. 7 Natural suppresor activity in bone marrow cells that had been incubated with LLC-CM is mediated predominantly by CD34+ cells. Normal bone marrow cells were cultured 3 days with LLC-CM. They were then either unfractionated, or divided into CD34+ and CD34− cell fractions. The capacity of these bone marrow fractions to inhibit splenic T cell proliferation in response to immobilized anti-CD3 was compared. Data shown are the mean absorbances ±SEM of four separate experiments

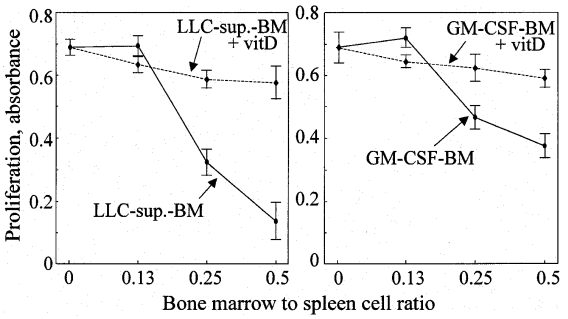

Fig. 8 1α,25-Dihdroxyvitamin D_3 [1,25$(OH)_2D_3$] (*vitD*) blocks the development of natural suppressor activity in LLC-CM-containing bone marrow cultures. Normal bone marrow cells were incubated for 8 days with LLC-CM (*left panel*), GM-CSF (*right panel*) or medium (not shown), in the presence or absence of 1,25$(OH)_2D_3$. The inhibitory effect of the bone marrow cells that had been incubated in the presence or absence of 1,25$(OH)_2D_3$ on splenic T cell proliferation (0.5:1 ratio) in response to immobilized anti-CD3 was compared. Data shown are the mean absorbances ±SEM of four separate experiments

The results of these studies show that culturing normal bone marrow cells with LLC-CM was sufficient to cause the normal bone marrow to acquire increased suppressive activity toward T cell activation through the TCR/CD3 complex, and that a significant level of this suppression could be attributed to the increased development of CD34+ cells. Since prior studies had shown that the capacity of tumors to induce CD34+ cells in vivo correlates with whether the tumor can produce GM-CSF, the present studies tested the extent to which GM-CSF contributed to the development of CD34+ cells. Also tested was whether GM-CSF was sufficient to stimulate the increase in CD34+ NS levels from normal bone marrow. These studies showed that GM-CSF was a necessary component of the LLC-CM for increasing CD34+ cell levels from normal bone marrow. In addition, culturing normal bone marrow with recombinant GM-CSF increased development of CD34+ NS cells. However, the levels of CD34+ cells and NS activity that were stimulated by GM-CSF were lower than those shown to be induced by the LLC-CM, even though the concentrations of GM-CSF used were similar to those that were in the LLC-CM. This suggests that tumor-derived cytokines other than GM-CSF contribute to the tumor stimulation of CD34+ NS. The identity of these additional cytokines has not yet been resolved. However, in prior studies with recombinant myelopoiesis-stimulatory cytokines, we determined that the induction of NS activity by GM-CSF from normal bone marrow could be synergistically enhanced by interleukin-3 (IL-3) [37]. While our preliminary studies have suggested that the LLC tumor cells produce IL-3, we have not yet tested the role of IL-3 within the LLC-CM in stimulating the increase in CD34+ cells from normal bone marrow.

The development of NS activity is not unique to LLC-LN7 tumor bearers. NS cells have been described in human cancers, animal tumor models, in non-tumor models where myelopoiesis is stimulated, and in normal human bone marrow [7, 11, 15, 24, 32]. For example, NS activity has been described during recovery from high-dose chemotherapy, and in the peripheral blood after mobilization with GM-CSF [7, 15]. Our studies have shown increased levels of CD34+ NS cells within the peripheral blood and within the cancer tissue of patients with GM-CSF-producing HNSCC [12, 42]. These CD34+ NS cells from either HNSCC patients or from mice with LLC-LN7 tumors mediate their NS activity predominantly through production of TGF-β [43, 44].

Although the LLC-CM caused a prominent expansion of CD34+ cells from normal bone marrow and the acquisition of potent NS activity, this was transient, with NS activity being prominent after 3 and 8 days of culture, and subsiding by 12 days. We speculated that this decline by 12 days of culture was probably due to the loss of CD34+ cells through their differentiation into nonsuppressive progeny cells. Therefore, the possibility was tested that it may be possible to block the development of NS activity with compounds that can induce myeloid differentiation. Our results supported this possibility by showing that the metabolically active hormone 1,25$(OH)_2D_3$, which has previously been shown to accelerate myeloid differentiation [10, 12, 33], reduced the expansion of CD34+ cells and, in turn, the development of NS activity. The characteristics of the differentiated progeny cells that result from the LLC-CM-containing bone marrow cells that had been cultured with 1,25$(OH)_2D_3$ have not been analyzed in the present studies. Nor has it been determined whether these cells are similar to those that would develop after exposure of bone marrow of tumor-bearing mice to 1,25$(OH)_2D_3$. Nevertheless, these studies have shown that tumor-derived soluble products induce the appearance of NS activity from the bone marrow of normal mice and that the NS activity is attributable to the expansion of CD34+ cells. GM-CSF is a significant contributor to this development of CD34+ NS cells, but perhaps it is not the only mediator of suppressor cell development. That 1,25$(OH)_2D_3$ can prevent this development of CD34+ NS activity can suggest strategies for blocking this immunosuppressive mechanism of tumors.

Acknowledgements We thank Ms. Rosann Grahovac for her help in the development of this manuscript. We would also like to thank Dr. Deanne Ridley Lathers for her contributions toward this study.

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